Exploration of the biomolecular approach of Indian Medicinal Plants for the application ofcardiotoxicity using *In-silico*, *In-vitro* and *In-vivo* approaches

A proposed synopsis report was submitted forpartial fulfillment of the degree of

Doctor of Philosophy

In

Pharmaceutical Sciences

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INTRODUCTION

Cardiovascular diseases (CVDs) are assessed to be the leading source of mortality and hospital admissions worldwide. The disrupted myocardial oxidants/antioxidants balance, the triggered inflammation, and the decreased cell viability influence cardiac function. Cardiotoxicity induced by drugs poses a serious risk to human health and is becoming an increasingly important concern in cardiooncology. Improvements in antineoplastic treatments led to increased overall and progression free survival in the management of an increasing number of malignancies. Cancer survivors, when compared with their healthy counterparts, are at high risk of cardiovascular-related deaths, including MI with coronary artery disease, cardiomyopathy with CHF,cerebrovascularevents.Cardiac toxic People receiving chemotherapy can be classified as having stage a heart failure. It is well known that chemotherapy drugs cause a wide range of toxicities, including heart damage. These may include heart failure, myocardial ischemia, arrhythmias, hypertension, myocarditis, pericarditis, and cardiac dysfunction leading to thromboembolism. Alkylating drugs, including cisplatin, cyclophosphamide, carmustine, chlormethine, busulfan, and mitomycin, are particularly associated with cardiotoxicity. (MulukenAltaye Ayza.et.al., 2020)

Cyclophosphamide (CP) is a drug widely used in cancer chemotherapy and immunosuppression that can induce toxicity in normal cells due to its toxic metabolites. Dose-dependent cardiotoxicity is a limiting factor for use. CP-induced cardiotoxicity is 7–28% and mortality is 11–43% at therapeutic doses of 170–180 mg/kg IV. CP is metabolized by the liver to produce aldophosphamide.Cyclophosphamide and its metabolites aldophosphamide, 4-hydroxycyclophosphamide and acrolein have been identified as cardiotoxic. In fact, acrolein is considered the most toxic metabolite.

Herbal medicines and their phytoconstituents are well-established alternative therapy in alleviating several acute and chronic ailments including CVDs.CVD and kidney disease are closely interrelated and disease of one organ cause dysfunction of the other, ultimately leading to the failure of both organs.

The overall quality of an herbal medicine may be affected by many factors, including seasonal changes, harvesting time, cultivation sites, post-harvesting processing, adulterants or substitutes of raw materials, and procedures in extraction and preparation. From harvesting to manufacturing, phytoconstituents play a crucial role in assessing the quality of herbal medicines (Alam et al., 2017). Moreover, the study of phytoconstituents is applicable to many research areas, including authentication, optimization of extraction and purification methods, structure elucidation and purity determination. Systematic investigations using chemical phytoconstituents may lead to development of new drugs (Zhang et al., 2018), (Bucar et al., 2013). Optimization of targeted bioactive phytoconstituents from an herbal medicine is one of the important step from separation science to biological science (Ahmad et al., 2020). Chromatography and spectroscopy techniques such as thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), Ultra performance liquid chromatography (UPLC), liquid chromatography and mass spectroscopy (LC-MS), gas chromatography and mass spectroscopy (GC-MS are mainly hyphenated with target based isolation, identification and quantitation of phytoconstituents from a huge diversity of herbal constituents as well as helps us to optimized and evaluate single constituents than complexity of phytoconstituents (Altemimi et al. 2017; Duarte et al. 2012).

With the development of the information age, various network technologies are constantly evolving. Network pharmacology combines network biology and multiplex pharmacology based on the low potency of highly selective single-targeted drugs. With the help of network pharmacology, we can directly identify drugs and disease targets from large amounts of data and understand the mechanisms and pathways between them. This is an efficient way. Currently, the scope of application of network pharmacology is expanding, including the study of the main pharmacological effects and mechanisms of drugs on diseases, the theory analysis of Chinese medicine, and the study of Chinese medicine use. With all these facts in mind, this study was designed to explore the application of network pharmacology by examining the metabolic patterns of several drugs and further studying them.

Literature Review

- Molecular mechanism of cyclophosphamide-induced cardiotoxicity: an old medicine with a fresh perspective was examined by **AshifIqubal*et al*.** in 2019. The study concluded that CP's metabolites cause cardiotoxicity because they lower antioxidant and ATP levels, change contractility, damage endothelium, and increase pro-inflammatory and pro-apoptotic activities, which lead to cardiomyopathy, myocardial infarction, and heart failure. The use of dosage adjustments, acrolein elimination or excretion, and preservation of the endogenous antioxidant pool might be used as a treatment strategy to reduce toxicity.

- Probucol Attenuates Cyclophosphamide-induced Oxidative Apoptosis, p53 and Bax Signal Expression in Rat Cardiac Tissues, according to research done by **Yousif A. Asiri (2010).** To sum up, the results of the present study indicate that probucol inhibits the development of CP-induced cardiotoxicity through a mechanism at least partially related to its capacity to boost antioxidant gene mRNA expression and reduce apoptosis in cardiac tissues, which leads to an improvement in mitochondrial oxidative phosphorylation and energy production.

- The Cardioprotective Efficacy of Croton macrostachyus Stem Bark Extract and Solvent Fractions on Cyclophosphamide-Induced Cardiotoxicity in Rats was assessed by **MulukenAltayeAyza*et al.* in 2020**. The findings show that Croton macrostachyus has cardioprotective properties and may be used to treat cardiotoxicity brought on by cyclophosphamide.

- **Somow S. Kamel.*et.al*., (2022)**studied the cardiotoxicityinduced by Cyclophosphamide.

- Nerolidol reduces the cyclophosphamide-induced cardiac inflammation, apoptosis, and fibrosis in Swiss Albino mice, according to a 2019 study by **AshifIqubal*et al***. The findings showed that nerolidol had powerful cardioprotective properties and reduced the cardiotoxicity caused by CP.

- The 2019 study Herbal Medication for Cardiovascular Diseases: Effectiveness, Mechanisms, and Safety by **Abdullah Shaito *et al***. In order to further understand the function of various medicinal plants and their underlying processes in relation to CVDs, the study indicated that additional clinical trials with bigger sample numbers and better-designed investigations are required. The safety and toxicity of these natural medicines should be the primary focus of next clinical research.

- **H. Avci et al (2017)** silymarin and curcumin's protective properties against cyclophosphamide-induced cardiotoxicity. The findings suggest that the natural antioxidants SLY and CUR may protect rats against cardiotoxicity and oxidative stress brought on by CP. The outcome supports the hypothesis that the natural antioxidants SLY and CUR may protect rats against cardiotoxicity and oxidative stress brought on by CP.

- The Cardioprotective Efficacy of Selenium Against Cyclophosphamide-Induced Cardiotoxicity in Rats was examined by **Sibel Gunes*et.al.***in **2016**. The findings lead to the suggestion that Se may have the capacity to reduce CP-induced cardiotoxicity, possibly due to its antioxidant action.

Drugs Possess Cardio Protective action		
Synthetic Drugs	**Crude Drugs**	
	Botanical Name	**Common Name**
Nerolidol	*Swertia chirayita*	Charaita
Silymarin	*Crategus hawthorns*	Thornapple
Nicorandil	*Damask rose*	Rose
Selenium	*Salvia coccinea*	Kamrkash
Allopurinol	*Salvia miltiorrhiza*	Red sage (Lal Bahaman)
Febuxostat	*Allium sativum*	Garlic

Aim & Objective

Aim:

Exploration of biomolecular approach of Indian Medicinal Plants for the treatment of cardiotoxicity using *In-silico*, *In-vitro* and *In-vivo* approaches.

Objectives:

- Collection and authentication of plant material as well as preparation of extract.
- Quality control analysis of extract using HPTLC/LC-MS/GC-MS analysis.
- *In-silico*/ Network pharmacology analysis to investigate biomolecular approaches of active principles for cardio protection.
- *In-vitro* anti-oxidant (DPPH, NO, ABTS and FRAP)
- *In-vivo* studies ofextract against cyclophosphamide induced cardiotoxicity.

Rationale of the study

Medicinal plants play an important role since the history to maintain the health and well-being of the humans. However, quality, safety, and efficacy based scientific validation of herbal drugs and their derived products still encroaching the need for further validation and to evade the negative perception among the society. Taking all these facts into consideration, in the present study, we explore the scientific evidences of biomolecular approaches to know about actual mechanism of action of Indian medicinal plants and their applications by recent analytical techniques and network pharmacology for generating scientific data for the validation of Indian herbal medicines.

Plan of work

Step 1: Collection & authentication of drug

- Selection of Drug
- Collection of Drug
- Authentication of Drug
- Extraction of Drug

Step 2: Identification of phytoconstituents

Quality control drugs via chromatographic techniques:

- HPTLC
- LC-MS or
- GC-MS

Step 3:*In-Silico* analysis using auto dock and cytoscape softwares

- Network biology analysis
- Network polypharmacology
- Gene ontology analysis (Metascape Gene Analysis tool)

Step 4:*In-vitro* Study

- Anti-Oxidant activity (DPPH, NO, ABTS and FRAP)

Step 5:*In-vivo* Study

- Toxicant used: cyclophosphamide

- Selection of drug (Standard & Test drug)
- Approval of experimental animal protocol
- Biochemical estimation for cardioprotective activity.
    - Anti-oxidant (SOD, CAD)
    - Anti-Inflammatory (TNF-alpha)
    - Lipid Profile (HDL, LDL, VLDL, Total cholesterol)
    - Cardiac markers (CK-MB, LDH, Trop-T)
- Histopathology analysis.

Material & Methods

Collection and authentication of plant material: Each plant material will be bought from vendors and validated utilizing the age-old procedures outlined in the UNANI pharmacopoeia of India. Each sample will be sent with a Boucher specimen to the pharmacy department at the IIMT University in Meerut.

Preparation of extracts: The ideal amount of each sample will be weighed, ground in the grinder, and then soaked in the appropriate solvent for extraction. After the procedure, the standard protocol's recommended extraction technique will be used, followed by filtering and concentration to provide a dry residue. Each extract's extractive yield will be determined and saved in airtight containers for further use.

Quality control of drugs

HPTLC profiling and quantitative estimation of major markers

Before being filtered via a PTFE membrane filter with a 0.2 M pore size, each extract will be individually diluted in the optimal quantity of HPLC grade methanol. Key indicators will be measured using the Camag TLC scanner III and the relevant wavelength of Wincats1.2.3 software.(Anjum et al., 2017)

LC-MS/MS analysis

Based on their molecular masses as determined from spectral data, the major and minor active metabolites will be identified via LC-MS/MS analysis. The samples' metabolomics will be analysed using the processed Mass Lynx V4.1 (Waters, USA) spectral data that was received from the MS. Separated metabolites found in various samples will be inferred based on their m/z value from literature and mass data sources including Mass Bank, PubChem, Drug Bank, and ChemSpider. (Khan et al., 2017),(Zahiruddin et al., 2017)

GC-MS analysis

GC-MS Agilent Technologies-7820A GC equipment will be used for the examination of plant drug's extracted phytochemicals. An Agilent Technologies GCMS capillary column HP-5MS made of 5% diphenyl 95% dimethyl poly siloxane is attached to the Agilent Technologies gas chromatograph paired with mass spectrometer, model 5977MSD. An electric field of 70 eV will be used to record mass spectra. The average peak area of each component will be compared to the total areas using the GC-MS Mass Hunter software, which is used to analyse the chromatograms and spectra.(N. Bhalla, N. Ingle, S.V. Patri et al., 2021)

Network pharmacology study

Network pharmacology analysis will be conducted to on identified metabolites of each plant to validate their therapeutic role, traditionally.

Selection of genes

Several genes will be selected based on their role in pathophysiology of the targeted disease. The selection and screening of genes with their protein name and specific ID will be performed form the GENE card data base and uniport ID data based. Each gene will be screened with their degree of ligation.

Inclusion and exclusion criteria of gene in network pharmacology

A protein-protein and protein-compound network will be conducted to determine the ligation effect of each gene. The genes which could not find with any interaction will be removed from the network during integration analysis of network. Afterall, the genes which would be found with the partial connection from each other, will be placed for final analysis.

Gene ontology and network construction of active compound and targets

The network pharmacology analysis predicted the ligation effectiveness of each chosen gene. After examining the compound-disease common target, a Gene Ontology (GO) study was carried out using the Metascape Gene Analysis (metascape.org) tool to assess the numerous physiological functions of each gene in the regulation of kidney and related illnesses. Based on the STRING platform (https://string-db.org/) and Cytoscape (version 3.8.2) software, a protein-protein interactions (PPI) network and compound-proteins interactions were discovered. For interaction information, integration, and further protein ligation building analysis, the target PPI was built and put into the Cytoscape programme. All the approximately functional connections between expressed proteins and proteins in complexes were examined. (Gaurav Gautam.et.al,2022) (Bharti Sharma.et.al,2022)

In-vitro Analysis

Anti-oxidant activity

DPPHfree radical scavenging activity will be conducted as per the reference procedure with some alteration(Gaurav *et al*., 2020). The anti-oxidant activity will be expressed in form of IC50 of the samples. Vitamin C will be used as the positive control. The measurements will be taken in triplicate and expressed statistically in form of the Mean ± SD using One-way or Two-way ANOVA followed by comparison of each row and columns.

Based on the DPPH scavenging activity, four potential plants will be screened based on their DPPH free radical inhibitory activity and will be used for further studies.

Nitric oxide (NO) anti-oxidant activitymeasurement:NO was estimated according to the method of Richa et al. (2017). (Richa et al., 2017).

ABTS and FRAP: These antioxidant parameterswill be conducted as per the reference protocol with some modification for in-vitro study.

The **FRAP assay** was conducted by (Benzie, et.al, 1996)&**ABTS assay**was determined by the method described by (Stratil.*et.al*.,2016&Natividad Chaves.*et. al* 2020)

***In-vivo* Study *(*Cyclophosphamide induced cardiotoxicity)**

Experimental animals All the experimental procedures involving the use of laboratory animals will be approved by the IAEC, IIMT University (India). Rats shall be Wistar albino males weighing 180-250 grammes from the Animal House Facility at the IIMT University in Meerut (India). For a week, the animals will be kept in polypropylene cages so they can become acclimated to the norm. (12 h light: dark cycle; 23 2 oC temperature; 60 % relative humidity). The animals were given a regular pellet meal and unrestricted access to the water supply.

Experimental design After acclimatization, the rats were divided into five groups (n=6) and treated as Group 1 (Normal control), Group 2 (Toxic control) and other Groups with Test drug. After 24 hours of the last dose, rats were sacrificed using ether anesthesia.

Cyclophosphamide

Cyclophosphamide (CP) is a drug that is frequently used as an immunosuppressive, neoplastic disease treatment, and oxazaphosphorine alkylating agent. CP is linked to serious drawbacks such damage to healthy tissue and possibly multiple organ poisoning. In addition to its negative impacts, the key factor is to maximise the hepatic microsomal cytochrome P450 mixed function oxidase system's effects on metabolic activation. At its high therapeutic dosages, CP is widely known to cause severe fatal cardiotoxicity, including myopericarditis or congestive heart failure, arrhythmias, cardiac tamponade, and myocardial depression.The development of acute cardiotoxicity and the production of free oxygen radicals, which compromise the heart's antioxidant defence mechanism, are linked to the pathophysiology of CP. The most often reported negative effects include hypertriglyceridemia, hypercholesterolemia, and impaired cardiac lipoprotein lipase secretion. CP is exponentially employed as the model of cardiotoxicity for in-vivo investigations and their respective assessment to identify the effect of test medicine against the CP-induced cardiotoxicity based on the facts.(Asiri, 2010).

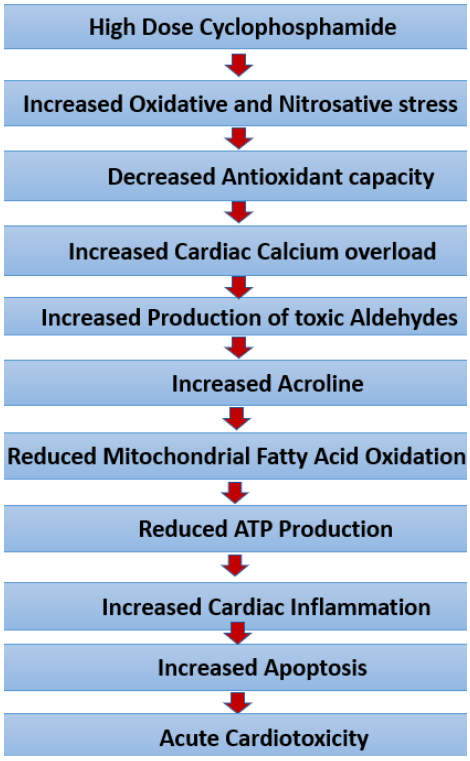

Fig: Cardiotoxicity caused by CP: molecular mechanisms

Toxicant used: Cyclophosphamide will be used as a toxicant.

Selection of drug (Standard & Test drug): Selection of test drug will be done based on recent research and review of literature after that more potent & drug having novelty will be taken for further study.

Approval of experimental animal protocol: Form-B will be filled with detailed explanation and procedure about the experiment design, dosage, with objective to IAEC (Institutional Animal Ethics Committee) for the approval of animals and further study.

Biochemical estimation for cardioprotective activity: The biochemical estimation will be done as per reference protocol with some modifications.

Anti-oxidant Estimation:

SOD- SOD activity was assessed using the Marklund and Marklund (1974) approach, which is based on the SOD enzyme's capacity to prevent pyrogallol autooxidation. SOD activity values were presented as U/mg of protein. (Marklund and Marklund, 1974).

CAT- Catalase activity was assessed according to the method of Claiborne, 1986 (Claiborne, 1986).

Anti-Inflammatory (TNF-alpha)- TNF- and IL-6 expression levels in the serum of all study groups of mice were measured using an ELISA kit in accordance with the manufacturer's instructions. Findings were given in pg/ml. (Gaurav.et.al,2021)

Lipid Profile (HDL, LDL, VLDL, Total cholesterol)- The lipid profile such as Total cholesterol (TC), Triglycerides (TG), high density lipoproteins (HDL) and low-density lipoproteins were estimated by standard methods. (Rifai N. *et.al,*1986)

Cardiac markers (CK-MB, LDH, Trop-T)-The estimation of marker enzymes was done using the separated serum. Troponin I was evaluated using a CS-200 acute care analyzer, whilst the BTS 350 semiautomated biochemistry analyzer examined the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (TC), and triglyceride (TG).(MulukenAltaye Ayza.et.al, 2020)

Histopathology analysis: The reference methodology will be modified slightly for the histopathology analysis. Animals' hearts were quickly removed, rinsed with water, dried with filter paper, weighed, and then preserved in 10% formalin for histological examination. Hematoxylin and eosin was used to stain the histological sections of the heart (H&E). Myocyte destruction, multifocal degeneration, inflammation, myofibrillar degeneration, and necrosis were all looked for in heart sections.

Statistical analysis

One-way ANOVA will be used to statistically represent the data as Mean SD (n=3), and the Tukey test will be used to compare each pair of columns.The significance level will be considered at $p<0.05$, and the comparison will be made between positive and negative control values throughout the experiments. Furthermore, the data obtained from the metabolomic studies will be expressed statistically using principal component analysis (PCA) and multivariate statistical analysis.

Timeline

Objectives	Time in months						
	1-3	3-6	6-9	9-12	12-15	15-18	18-24
Collection and authentication of plant material	■						
Quality control analysis of drug using HPTLC/LCMS/GCMS analysis		■					
In-silico/ Network pharmacology analysis to investigate biomolecular approaches of active principles for CVD			■				
In-vitro anti-oxidant & Lipid profiling				■			
In-vivo studies of extract against cyclophosphamide induced cardiotoxicity					■	■	
Data compilation and publication		■	■	■	■	■	■

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